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THE INCIDENCE OF FUNGI AND MYCOTOXINS ASSOCIATED
WITH STARCH-BASED FOOD STORED AT DIFFERENT
LEVELS OF WATER ACTIVITY

BY

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ABSTRACT

The incidence of fungi and mycotoxin production in ordinary rice grains, glutinous rice grains, riceflour, glutinous riceflour, wheatflour and cornflour stored at different levels of A_w were investigated. In a survey of starch-based food sampled from retail outlets in Malaysia, fungal colonies were mostly detected in wheatflour (100%), followed by riceflour (74%), glutinous rice grains (72%), ordinary rice grains (60%), glutinous riceflour (48%) and cornflour (26%). All positive samples of ordinary rice and glutinous rice grains had total mycoflora count below 10^3 cfug⁻¹ sample, while among the riceflour, glutinous riceflour and cornflour samples, 2%, 10% and 4% had more than 10^3 but less than 10^4 cfug⁻¹ sample respectively. Sixteen per cent of wheatflour samples had more than 10^3 cfug⁻¹ sample and 2% had more than 10^4 cfug⁻¹ sample. Aflatoxigenic colonies were mostly detected in wheatflour (20%) samples, followed by ordinary rice grains (4%) and glutinous rice grains (4%) and glutinous riceflour (2%). Samples of riceflour and cornflour were absent from aflatoxigenic colonies. Screening of aflatoxin B₁, aflatoxin B₂, aflatoxin G₁ and aflatoxin G₂ using reversed-phase HPLC were carried out on 84 samples of ordinary rice grains and 83 samples of wheatflour. Two point four percent (2.4%) of ordinary rice grains were positive for aflatoxin G₁ and 3.6% were positive for aflatoxin G₂. All the positive samples were collected from private homes at concentrations ranging from 3.69 μgkg^{-1} - 77.50 μgkg^{-1} . One point two percent (1.2%) of wheatflour samples were positive for aflatoxin B₁ at a concentration of 25.62 μgkg^{-1} , 4.8% were positive for aflatoxin B₂ at concentrations ranging from 11.25 - 252.50 μgkg^{-1} , 3.6% were positive for aflatoxin G₁ at concentrations ranging from 25.00 - 289.38 μgkg^{-1} and 13.25% were positive for aflatoxin G₂ at concentrations ranging from 16.25 - 436.25 μgkg^{-1} . Similarly, positive wheatflour samples were mostly collected from private homes.

From the water vapour adsorption isotherms, the critical moisture content (% dry basis) i.e. the moisture content to be maintained at 25 °C that will not allow fungal growth was found to be 13.01% for ordinary rice grains, 12.87% for glutinous rice grains, 9.56% for riceflour, 10.61% for glutinous riceflour, 10.72% for wheatflour and 10.52% for cornflour. Correspondingly, in order to maintain

these moisture contents levels, starch-based food must be stored at water activity level of not more than 0.65 equivalent to an equilibrium relative humidity of 65%. However, from this study, at 0.65 A_w , visible appearance of fungi still occurred after 57 days for ordinary rice grains and 73 days for glutinous rice grains. Hence, for ordinary rice and glutinous rice grains, a moisture content lower than 13.01% and 12.87% must be maintained respectively for long-term storage. As water activity of starch-based food increases the development of fungi occurred earlier.

In this study, storage of starch-based food at different levels of water activity at 25 °C for 96 days indicate that the most common genera isolated were *Penicillium* and its teleomorphs (22 species) and *Aspergillus* and its teleomorphs (13 species). The others were zygomycetes (6 species) and *Curvularia lunata*, *Dreschlera* sp., *Moniliella* sp., *Monascus mucoroides* and *Trichoderma* sp. The dominant fungal species occurring on ordinary rice grains were *A. candidus*, *A. flavus*, *A. niger*, *Rhizopus arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. candidus* occurred at water activity levels of 0.65, 0.80, 0.85 and 0.98, by *A. flavus* at 0.75 A_w and by *R. arrhizus* at 0.75 and 0.90 A_w . The dominant fungal species occurring on glutinous rice grains were *A. flavus*, *A. niger*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* occurred at 0.75 A_w and by *R. microsporus* at all water activities except 0.85. The dominant fungal species occurring on riceflour were *A. flavus*, *A. niger*, *A. terreus*, *P. chrysogenum*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* and *R. arrhizus* occurred at water activity levels of 0.65 - 0.90, by *A. niger* at 0.90 A_w , by *A. terreus* and *P. chrysogenum* at all water activity levels and by *R. microsporus* at 0.75 and 0.85 A_w . The dominant fungal species occurring on glutinous riceflour were *A. flavus*, *A. niger*, *P. chrysogenum*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* occurred at all water activity levels except at 0.95, by *A. niger* at all water activity levels, by *P. chrysogenum* at 0.80 - 0.98 A_w , by *R. arrhizus* at 0.80 and 0.85 A_w and by *R. microsporus* at 0.90 A_w . The dominant fungal species occurring on wheatflour were *A. candidus*, *A. flavus* and *P. chrysogenum*. A significant increase in the incidence from day 0 by *A. candidus* occurred at all water activity levels, by *A.*

flavus at 0.75 and 0.80 A_w and by *P. chrysogenum* at 0.65, 0.80, 0.85, 0.90 and 0.98 water activity levels. Only *P. chrysogenum* was dominant on cornflour and a significant increase in the incidence from day 0 occurred at all water activity levels.

The dominant fungal species isolated from starch-based food played an important role in the biodeterioration of starch-based food since they have high growth rates ranging from 2.4 mmday⁻¹ colony diameter for *A. terreus* to 31.8 mmday⁻¹ colony diameter for *R. microsporus*. *Rhizopus arrhizus* was the fastest growing, covering the petri dish in 2 days on starch agar at 25 °C. Furthermore, most of these species were found to exhibit amylolytic activity hence capable of degrading starch.

Mycotoxin extraction and detection, by reversed-phase HPLC using C₁₈ column and elution solvent consisting of water and 0.05% TFA in acetonitrile, was evaluated. The extraction method gave good recoveries i.e > 90% for patulin, > 91% for aflatoxin G₁, > 92% for aflatoxin B₁, > 83% for griseofulvin, > 89% for cytochalasin E, > 88% for ochratoxin A and > 90% for sterigmatocystin. The reversed-phase HPLC was able to detect and quantitate seven mycotoxins in 34 min and the retention times obtained was highly reproducible. It was most sensitive for patulin, griseofulvin, aflatoxin G₁, sterigmatocystin, griseofulvin, aflatoxin B₁ and ochratoxin A being able to detect nanogram amounts of the toxin.

Storage of starch-based food at different levels of water activities resulted in the production of various types of mycotoxins at A_w above 0.80 and usually after a period of 26 days and above. Aflatoxin B₁ was the most common mycotoxin present in the starch-based food studied.

Among the starch-based food studied, the highest toxicity was exhibited by glutinous riceflour stored at 0.98 A_w for 54 days, followed by wheatflour stored at 0.90 A_w for 96 days, followed by ordinary rice grains stored at 0.95 A_w for 96 days, followed by riceflour stored at 0.95 A_w for 54 days, followed by glutinous rice grains stored at 0.85 A_w for 96 days and lastly followed by cornflour stored at 0.90 A_w for 54 days.

ABSTRAK

Kehadiran kulat dan pengeluaran mikotoksin pada beras, beras pulut, tepung beras, tepung beras pulut, tepung gandum dan tepung jagung yang disimpan pada paras aktiviti air (A_w) yang berlainan telah dikaji. Di dalam satu tinjauan makanan berasaskan kanji, pensampelan dari kedai-kedai runcit di Malaysia, menunjukkan koloni kulat paling kerap tumbuh pada tepung gandum (100%), di ikuti dengan tepung beras (74%), beras pulut (72%), beras (60%), tepung beras pulut (48%) dan tepung jagung (26%). Kesemua sampel beras dan beras pulut yang positif mempunyai jumlah kiraan mikoflora kurang daripada 10^3 cfug⁻¹ sampel, manakala di antara sampel-sampel tepung beras, tepung beras pulut dan tepung jagung, 2%, 10% dan 4% mempunyai lebih daripada 10^3 tetapi kurang daripada 10^4 cfug⁻¹ sampel masing-masing. Enam-belas peratus sampel tepung jagung mempunyai lebih daripada 10^3 cfug⁻¹ sampel dan 2% mempunyai lebih daripada 10^4 cfug⁻¹ sampel. Koloni-koloni kulat aflatoksigenik paling kerap tumbuh pada sampel tepung gandum (20%), diikuti dengan sampel beras (4%) dan beras pulut (4%) dan tepung beras pulut (2%). Koloni kulat aflatoksigenik tidak tumbuh pada sampel-sampel tepung beras dan tepung jagung. Penskrinan aflatoxin B₁, aflatoxin B₂, aflatoxin G₁ dan aflatoxin G₂ menggunakan 'reversed-phase' HPLC telah dilakukan ke atas 84 sampel beras dan 83 sampel tepung gandum. Dua perpuhan empat peratus (2.4%) sampel beras di dapati positif mengandungi aflatoxin G₁ dan 3.6% adalah positif mengandungi aflatoxin G₂. Kesemua sampel positif dikutip daripada rumah persendirian dalam julat kepekatan 3.69 - 77.50 μgkg^{-1} . Satu perpuhan dua peratus (1.2%) sampel tepung gandum di dapati positif mengandungi aflatoxin B₁ dalam kepekatan

25.62 μgkg^{-1} , 4.8% adalah positif mengandungi aflatoksin B_1 dalam julat kepekatan 11.25 - 252.50 μgkg^{-1} , 3.6% adalah positif mengandungi aflatoksin G_1 dalam julat kepekatan 25.00 - 289.38 μgkg^{-1} dan 13.25% adalah positif mengandungi aflatoksin G_2 dalam julat kepekatan 16.25 - 436.25 μgkg^{-1} . Sampel-sampel tepung gandum yang positif juga di kutip daripada rumah persendirian.

Daripada isoterma jerapan wap air (water vapour adsorption isotherm), kandungan kelembapan yang kritikal (% kering) i.e. kandungan kelembapan yang mesti dikekalkan pada suhu 25 °C yang tidak membolehkan pertumbuhan kulat pada beras adalah 13.01%, pada beras pulut adalah 12.87%, pada tepung beras adalah 9.56%, pada tepung beras pulut adalah 10.61%, pada tepung gandum adalah 10.72% dan pada tepung jagung adalah 10.52%. Selari dengan ini, untuk mendapatkan kandungan kelembapan di atas, makanan berasaskan kanji mesti disimpan pada aktiviti air tidak melebihi 0.65 setara dengan kelembapan bandingan keseimbangan 65%. Tetapi daripada kajian ini, pada A_w 0.65, kulat mula kelihatan dengan nyata selepas 57 hari untuk beras dan 73 hari untuk beras pulut. Oleh itu, untuk beras dan beras pulut, kandungan kelembapan kurang daripada 13.01% dan 12.87% masing-masing mesti dikekalkan untuk penyimpanan jangka panjang. Perkembangan kulat terjadi dengan lebih cepat apabila penyimpanan pada paras aktiviti air meningkat.

Dalam kajian ini, penyimpanan makanan berasaskan kanji pada paras aktiviti air berlainan pada suhu 25 °C untuk 96 hari menunjukkan genera yang paling kerap dipencil adalah *Penicillium* dan teleomofanya (22 spesies) dan *Aspergillus* dan teleomofanya (13 spesies). Kulat yang lain adalah zigomiset (6 spesies) dan *Curvularia lunata*, *Dreschlera* sp., *Moniliella* sp., *Monascus*

mucoroides dan *Trichoderma* sp. Spesies kulat yang dominan pada beras adalah *A. candidus*, *A. flavus*, *A. niger*, *Rhizopus arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. candidus* berlaku pada aktiviti air 0.65, 0.80, 0.85 dan 0.98, oleh *A. flavus* pada A_w 0.75 dan oleh *R. arrhizus* pada aktiviti air 0.75 dan 0.90. Spesies kulat yang dominan pada beras pulut adalah *A. flavus*, *A. niger*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* berlaku pada A_w 0.75 dan oleh *R. microsporus* pada semua paras aktiviti air melainkan 0.85. Species kulat yang dominan pada tepung beras adalah *A. flavus*, *A. niger*, *A. terreus*, *P. chrysogenum*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* dan *R. arrhizus* berlaku pada paras aktiviti air 0.65 - 0.90, oleh *A. niger* pada A_w 0.90, oleh *A. terreus* dan *P. chrysogenum* pada kesemua paras aktiviti air dan oleh *R. microsporus* pada A_w 0.75 dan 0.85. Spesies kulat yang dominan pada tepung beras pulut adalah *A. flavus*, *A. niger*, *P. chrysogenum*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* berlaku pada kesemua paras aktiviti air melainkan 0.95, *A. niger* pada kesemua paras aktiviti air, oleh *P. chrysogenum* pada A_w 0.80 - 0.98, oleh *R. arrhizus* pada 0.80 dan 0.85 dan oleh *R. microsporus* pada A_w 0.90. Spesies kulat yang dominan pada tepung gandum adalah *A. candidus*, *A. flavus* dan *P. chrysogenum*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. candidus* berlaku pada kesemua paras aktiviti air, oleh *A. flavus* pada A_w 0.75 dan 0.80 dan oleh *P. chrysogenum* pada paras aktiviti air 0.65, 0.80, 0.85, 0.90 dan 0.98. Hanya *P. chrysogenum* yang dominan pada tepung jagung dan

perbezaan peningkatan kehadiran yang nyata daripada hari 0 berlaku pada semua paras aktiviti air.

Kulat-kulat yang dominan pada makanan berasaskan kanji memainkan peranan yang penting dalam biopereputan oleh kerana ia mempunyai kadar pertumbuhan yang tinggi di antara 2.40 mmhari⁻¹ diameter koloni untuk *A. terreus* sehingga 31.83 mmhari⁻¹ diameter koloni untuk *R. microsporus*. *Rhizopus arrhizus* mempunyai kadar pertumbuhan paling tinggi dengan memenuhi piring petri dalam masa 2 hari atas agar kanji pada suhu 25 °C. Kebanyakan spesies ini di dapati mempunyai aktiviti amilolitik iaitu boleh menghurai bahan kanji.

Kaedah pengekstrakan dan pengesanan mikotoksin dengan 'reversed-phase' HPLC menggunakan kolum C₁₈ dan pelarut elusi yang terdiri daripada air dan 0.05% TFA dalam asetonitril telah dinilai. Kaedah pengekstrakan yang digunakan memberi penghasilan balik yang tinggi i.e. > 90% untuk patulin, > 91% untuk aflatoksin G₁, > 92% untuk aflatoksin B₁, > 83% untuk griseofulvin, > 89% untuk cytochalasin E, > 88% untuk okratoksin A dan > 90% untuk sterigmatosistin. HPLC 'reversed-phase' boleh mengesan dan kuantitat tujuh mikotoksin dalam masa 34 min dan masa retensi (retention time) yang di dapati adalah boleh ulang. Ianya paling sensitif untuk patulin, griseofulvin, aflatoksin G₁, sterigmatosistin, griseofulvin, aflatoksin B₁ dan okratoksin A iaitu boleh mengesan sehingga nanogram toksin.

Penyimpanan makanan berasaskan kanji pada paras aktiviti air yang berlainan menyebabkan pengeluaran beberapa jenis mikotoksin pada A_w lebih daripada 0.80 pada jangkamasa lebih daripada 26 hari. Aflatoksin B₁ adalah paling kerap hadir dalam makanan berasaskan kanji yang dikaji.

Di antara makanan berasaskan kanji yang dikaji, ketoksikan yang paling tinggi ditunjukkan oleh tepung beras pulut setelah disimpan pada A_w 0.98 selama 54 hari, di ikuti dengan tepung gandum setelah disimpan pada A_w 0.90 selama 96 hari, di ikuti dengan beras setelah disimpan pada A_w 0.95 selama 96 hari, di ikuti dengan tepung beras setelah disimpan pada A_w 0.95 selama 54 hari, di ikuti dengan beras pulut setelah disimpan pada A_w 0.85 selama 96 hari dan akhir sekali oleh tepung jagung setelah disimpan pada A_w 0.90 selama 54 hari.

TABLE OF CONTENTS	PAGE
Acknowledgement	i
Abstract	ii
Abstrak	v
List of Tables	xiv
List of Figures	xviii
List of Plates	xx
Abbreviations	xxi

Chapter 1.0 General Introduction

1.1 Basic concepts of water availability in food	1
1.1.1 Water activity (A_w)	1
1.1.2 Sorption effects	2
1.2 Factors affecting fungal growth and mycotoxin production in food	4
1.2.1 Water activity	4
1.2.2 Temperature	5
1.2.3 Interaction of temperature and water activity	6
1.2.4 pH	8
1.2.5 Oxygen (and carbon dioxide) tension	8
1.2.6 Type of substrate and nutritional factors	9
1.2.7 Consistency	10
1.2.8 Chemical treatment and presence of preservatives	11
1.2.9 Specific solute effects	12
1.3 Mycotoxins	12
1.3.1 Definition	12
1.3.2 History of mycotoxins	13
1.3.3 Structure and formation of mycotoxins	14
1.3.4 Mycotoxins	19
1.3.5 Toxicological impact of important mycotoxins	21
1.4 Mycotoxins produced by species of <i>Penicillium</i> and <i>Aspergillus</i> occurring on cereals	26
1.5 Present status of fungal contamination and mycotoxin situation in Malaysia	30
1.6 Scope and objectives of study	34

	PAGE
Chapter 2.0 Survey of fungal counts and the incidence of aflatoxin producing species in starch-based food and screening for aflatoxins in ordinary rice and wheatflour at the consumer level in Malaysia	
2.1 Introduction	37
2.2 Materials and methods	39
2.2.1 Sampling of starch-based food	39
2.2.2 Determination of fungal counts in starch-based food by dilution plating	40
2.2.3 Extraction and analysis of aflatoxins	41
2.3 Results	42
2.3.1 Total mycoflora count and aflatoxin producing colonies	42
2.3.2 Resolution of aflatoxin B ₁ , aflatoxin B ₂ , aflatoxin G ₁ – and aflatoxin G ₂	47
2.3.3 Aflatoxin contamination in rice and wheatflour samples	50
2.4 Discussion	54
Chapter 3.0 Water adsorption isotherms and fungal development on starch-based food stored at different levels of water activity	
3.1 Introduction	57
3.2 Materials and methods	59
3.2.1 Sampling and subsampling of starch-based food	59
3.2.2 Determination of the initial moisture content of starch-based food	59
3.2.3 Determination of the water adsorption isotherm of starch-based food	60
3.2.4 Fungal development in starch-based food stored at various levels of water activity	60
3.3 Results	61
3.3.1 The initial moisture content of starch-based food	61
3.3.2 Water adsorption isotherm of starch-based food	61
3.3.3 Time taken for the development of fungi in starch-based food stored at various levels of water activity at 25 °C	66

	PAGE
3.4 Discussion	68
Chapter 4.0 The incidence of fungi in starch-based food stored at different levels of water activity and their role in biodeterioration	
4.1 Introduction	72
4.2 Materials and method	75
4.2.1 Fungal incidence on starch-based food stored at different levels of water activity	75
4.2.1.1 Incubation of starch-based food	75
4.2.1.2 Direct plating method	75
4.2.1.3 Dilution plating method	75
4.2.1.4 Media used for the enumeration of fungi	76
4.2.1.5 Incubation and analysis of plates	76
4.2.2 Participatory roles of isolates in biodeterioration of starch-based food	77
4.3 Results	78
4.4 Discussion	104
Chapter 5.0 Descriptions of species isolated	
5.1 List of the species isolated from starch-based food	109
5.2 Descriptions of the species	110
5.3 Colony Plates	136
5.4 Light microscope and scanning electron microscope (SEM) plates of fungi from starch-based food	158
Chapter 6.0 Keys	
6.0 Key to genera of fungi isolated from starch-based food	181
6.1 General key to starch-based food fungi	180
6.2 Microscopic key to the genera Mucorales	180
6.3 Key to <i>Aspergillus</i> species and teleomorphs	181
6.4 Key to <i>Penicillium</i> species and teleomorphs	182
Chapter 7.0 Biochemical studies of starch-based food after storage at different levels of water activity	
7.1 Introduction	186

	PAGE
7.2 Materials and methods	192
7.2.1 Extraction and reversed phase HPLC methods for detection and quantitation of mycotoxins	192
7.2.1.1 Apparatus	192
7.2.1.2 Reagents	193
7.2.1.3 Sample preparation	193
7.2.1.4 Extraction and cleanup	194
7.2.1.5 Recovery and reproducibility studies	194
7.2.1.6 Mycotoxin standard solutions	195
7.2.1.7 HPLC analyses	196
7.2.2 The production of mycotoxins in starch-based food stored at different levels of water activity	197
7.2.2.1 Incubation of starch-based food	197
7.2.2.2 Analysis for mycotoxins	197
7.2.3 Toxicity of starch-based food extracts on the brine shrimp, <i>Artemia salina</i> L.	197
7.2.3.1 Breeding of the larvae	- 197
7.2.3.2 Method of testing	198
7.2.3.3 Analysis of bioassay data	199
7.3 Results	199
7.3.1 Resolution of seven mycotoxins by reversed-phase HPLC	199
7.3.2 Recovery and reproducibility studies	203
7.3.3 Quantitation of mycotoxins in starch-based food stored at different levels of water activity	205
7.3.4 Toxicity of starch-based food extracts on the brine shrimp, <i>Artemia salina</i> L.	214
7.4 Discussion	225
Chapter 8.0 General discussion	231
Chapter 9.0 References	243
Chapter 10.0 Appendices	
Appendix A: Culture media	265
Appendix B: Analytical methods	268
Appendix C: Newspaper reports on mycotoxin incident in Malaysia	274
Appendix D: Experimental data	282

LIST OF TABLES	PAGE
1.1 Polyketide-derived mycotoxins	16
1.2 A selection of trichothecenes and their sources	17
1.3 Potential mycotoxin production by important species of <i>Aspergillus</i> and teleomorphs occurring on cereals	28
1.4 Potential mycotoxin production by important species of <i>Penicillium</i> and teleomorph occurring on cereals	29
2.1 Total mycoflora counts (cfu per gram sample) and aflatoxins producing colonies on ordinary rice and glutinous rice samples collected from retail outlets	43
2.2 Total mycoflora counts (cfu per gram sample) and aflatoxins producing colonies on riceflour and glutinous riceflour samples collected from retail outlets	44
2.3 Total mycoflora counts (cfu per gram sample) and aflatoxins producing colonies on wheatflour and cornflour samples collected from retail outlets	46
2.4 Reproducibility of retention times of aflatoxins by HPLC with coefficient of variation	47
2.5 Peak height reproducibility in the separation of aflatoxins by HPLC	49
2.6 Detection limit and sensitivity of aflatoxins by HPLC	49
2.7 Distribution of aflatoxins in ordinary rice grains collected in Malaysia	50
2.8 Concentration of aflatoxins detected in positive ordinary rice samples	51
2.9 Distribution of aflatoxins in wheatflour collected in Malaysia	52
2.10 Concentration of aflatoxins detected in positive wheatflour samples	53
3.1 The initial moisture content of the composite samples of each starch-based food (% dry basis)	61
3.2 Equilibrium moisture content of starch-based food at different water activities at 25 °C	62

	PAGE
3.3 Days before visible appearance of fungi on six starch-based food stored at 25 °C	66
4.1 The incidence of fungi on composite samples of starch-based food before storage (Day 0) at different levels of water activity	79
4.2 The incidence of fungi on ordinary rice stored at different levels of A_w at 25 °C for 96 days	83
4.3 The incidence of fungi on glutinous rice stored at different levels of A_w at 25 °C for 96 days	85
4.4 The incidence of fungi on riceflour stored at different levels of A_w at 25 °C for 96 days	88
4.5 The incidence of fungi on glutinous riceflour stored at different levels of A_w at 25 °C for 96 days	90
4.6 The incidence of fungi on wheatflour stored at different levels of A_w at 25 °C for 96 days	93
4.7 The incidence of fungi on cornflour stored at different levels of A_w at 25 °C for 96 days	95
4.8 Fungal growth rates, amylolytic activity and changes they induced on starch-extract agar after 7 days of growth at 25 °C	102
7.1 Reproducibility of retention times for seven mycotoxins by HPLC with coefficient of variation	201
7.2 Peak height reproducibility in the separation of seven mycotoxins by HPLC	202
7.3 Detection limit and sensitivity of mycotoxins by HPLC	203
7.4 Recovery and reproducibility data for mycotoxins in spiked starch-based food	204
7.5 The incidence of mycotoxins (μgg^{-1}) in starch-based food stored at different levels of A_w at 25 °C	207
7.6 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against ordinary rice extracts stored at different levels of A_w	218

	PAGE
7.7 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against glutinous rice extracts stored at different levels of A_w	219
7.8 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against riceflour extracts stored at different levels of A_w	220
7.9 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against glutinous riceflour extracts stored at different levels of A_w	222
7.10 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against wheatflour extracts stored at different levels of A_w	223
7.11 Computed probit analyses data (18 hrs) obtained for brine shrimp — larvae, tested against cornflour extracts stored at different levels of A_w	224
B3.1 Concentration of sodium hydroxide giving specified Vapour pressure at 25 °C	268
D2.1 Data for retention times of aflatoxins B ₁ , B ₂ , G ₁ , and G ₂ obtained by six successive injections of single and mixed aflatoxin standards at 0.10 and 0.15 mgml ⁻¹ over 4 days detected by HPLC using acetonitrile : methanol : water (1 : 1 : 2)	282
D2.2 Data for peak heights reproducibility of aflatoxins obtained by six successive injections of mixed aflatoxins standards at 0.10 and 0.15 mgml ⁻¹ over 2 days detected by HPLC using acetonitrile : methanol : water (1 : 1 : 2)	282
D3.1 Equilibrium moisture content of starch-based food at different water activity at 25 °C	283
D3.2 Days before visible appearance of fungi on starch-based food stored at different levels of water activity at 25 °C	283
D4.1 Incidence of fungi on starch-based food at various level of water activities	284
D4.2 The percentage incidence (% of the total no. of plates fungal species occurred/total no. of plates) of dominant fungal species on starch-based food at each level of water activity over a period of 96 days at 25 °C	326

D4.3	Growth rate (mmday^{-1}) colony diameter during linear phase of growth at 25 °C	327
D7.1	Data for retention times of seven mycotoxins by reversed-phase HPLC obtained by seven successive injections of single and mixed mycotoxin standards over four days	328
D7.2	Data for peak heights of seven mycotoxins by reversed-phase HPLC obtained by seven successive injections of mixed mycotoxin standards ranging from 0.1 mgml^{-1} - 1.6 mgml^{-1} over two days	328

LIST OF FIGURES	PAGE
1.1 Relationship between moisture content and A_w value for foods showing typical hysteresis loop consisting of adsorption and desorption isotherms	3
1.2 Intermediates linking primary and secondary metabolism	15
1.3 The nonadrides	18
1.4 Primary and secondary mycotoxins	20
2.1 HPLC of a mixture of aflatoxins B ₁ , B ₂ , G ₁ and G ₂	48
3.1 Water adsorption isotherm of ordinary rice grains at 25 °C	63
3.2 Water adsorption isotherm of glutinous rice grains at 25 °C	63
3.3 Water adsorption isotherm of riceflour at 25 °C	64
3.4 Water adsorption isotherm of glutinous riceflour at 25 °C	64
3.5 Water adsorption isotherm of wheatflour at 25 °C	65
3.6 Water adsorption isotherm of cornflour at 25 °C	65
3.7 Relationship between water activity and the time before visible appearance of fungi on starch-based food at 25 °C	67
4.1 Changes in incidence of dominant species of fungi on ordinary rice stored at different levels of water activity at 25 °C	98
4.2 Changes in incidence of dominant species of fungi on glutinous rice stored at different levels of water activity at 25 °C	98
4.3 Changes in incidence of dominant species of fungi on riceflour stored at different levels of water activity at 25 °C	99
4.4 Changes in incidence of dominant species of fungi on glutinous riceflour stored at different levels of water activity at 25 °C	99
4.5 Changes in incidence of dominant species of fungi on wheatflour stored at different levels of water activity at 25 °C	100
4.6 Changes in incidence of dominant species of fungi on cornflour stored at different levels of water activity at 25 °C	100
7.1 HPLC of a mixture of seven mycotoxins	200

	PAGE
7.2 HPLC of starch-based food at day 0	206
7.3 HPLC of ordinary rice grains stored at 0.90 A_w at 25 °C for 96 days	209
7.4 HPLC of glutinous rice grains stored at 0.95 A_w at 25 °C for 26 days	211
7.5 HPLC of riceflour stored at 0.90 A_w at 25 °C for 54 days	212
7.6 HPLC of glutinous riceflour stored at 0.95 A_w at 25 °C for 54 days	213
7.7 HPLC of wheatflour stored at 0.85 A_w at 25 °C for 26 days	215
7.8 HPLC of cornflour stored at 0.90 A_w at 25 °C for 54 days	216
D7.3 Chromatograms of starch-based food stored at different levels of water activity at 25 °C	329

LIST OF PLATES	PAGE
4.1 Strongly amylolytic fungi isolated from starch-based food	103
4.2 Moderately amylolytic fungi isolated from starch-based food	103
4.3 Non-amylolytic fungi isolated from starch-based food	104
5.3 Colony plates	136
5.4 Light microscope and scanning electron microscope (SEM) plates of fungi from starch-based food	158

LIST OF ABBREVIATIONS

A_w	: water activity
ATA	: Alimentary Toxic Aleukia
BET	: Brunauer-Emmett Teller
cfu	: colony forming units
cfug^{-1}	: colony forming units per gram
CCL_4	: carbon tetrachloride
CDA	: Czapek-dox agar
CHCl_3	: chloroform
CO_2	: carbon dioxide
$^{\circ}\text{C}$: degree Celcius
ERH	: Equilibrium relative humidity
g	: grams
HPLC	: High Performance Liquid Chromatography
hrs	: hours
IARC	: International Agency for Research on Cancer
ICMSF	: International Commission on Microbiological Specification for Foods
KNO_3	: potassium nitrate
LC_{50}	: fifty percent lethal concentration
MARDI	: Malaysian Agricultural Research and Development Institute
MEA	: Malt extract agar
μgg^{-1}	: microgram per gram
μgkg^{-1}	: microgram per kilogram
μgml^{-1}	: microgram per millilitre
μl	: microlitre
μm	: micrometre
mins	: minutes
mgml^{-1}	: milligram per millilitre
mlmin^{-1}	: millilitre per minute
mm	: millimetre
mmday^{-1}	: millimetre per day
$(\text{NH}_4)\text{SO}_4$: ammonium sulphate
O_2	: oxygen
PDA	: Potato dextrose agar
S.E.	: standard error
TFA	: Trifluoroacetic acid
U.S.A.	: United States of America
Vps	: vapour pressure of water in food
Vpw	: vapour pressure of pure water
WHO	: World Health Organisation